

Effect of Phorbol 12-Myristate 13-Acetate on the Differentiation of Adipose-Derived Stromal Cells from Different Subcutaneous Adipose Tissue Depots

Jennifer K. Song¹, Chang Hoon Lee², So-Min Hwang¹, Bo Sun Joo³, Sun Young Lee⁴, and Jin Sup Jung⁴

¹Aesthetic, Plastic, & Reconstructive Surgery Center, Good Moonhwa Hospital, ²S&M Research Institute, Good Moonhwa Hospital, ³Center for Reproductive Medicine, Good Moonhwa Hospital, Busan 614-847, ⁴Department of Physiology, School of Medicine, Pusan National University, Yangsan 626-790, Korea

Human adipose-tissue-derived stromal cells (hADSCs) are abundant in adipose tissue and can differentiate into multi-lineage cell types, including adipocytes, osteoblasts, and chondrocytes. In order to define the optimal harvest site of adipose tissue harvest site, we isolated hADSCs from different subcutaneous sites (upper abdomen, lower abdomen, and thigh) and compared their proliferation and potential to differentiate into adipocytes and osteoblasts. In addition, this study examined the effect of phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, on proliferation and differentiation of hADSCs to adipocytes or osteoblasts. hADSCs isolated from different subcutaneous depots have a similar growth rate. Fluorescence-activated cell sorting (FACS) analysis showed that the expression levels of CD73 and CD90 were similar between hADSCs from abdomen and thigh regions. However, the expression of CD105 was lower in hADSCs from the thigh than in those from the abdomen. Although the adipogenic differentiation potential of hADSCs from both tissue regions was similar, the osteogenic differentiation potential of hADSCs from the thigh was greater than that of hADSCs from the abdomen. Phorbol 12-myristate 13-acetate (PMA) treatment increased osteogenic differentiation and suppressed adipogenic differentiation of all hADSCs without affecting their growth rate and the treatment of Go6983, a general inhibitor of protein kinase C (PKC) blocked the PMA effect. These findings indicate that the thigh region might be a suitable source of hADSCs for bone regeneration and that the PKC signaling pathway may be involved in the adipogenic and osteogenic differentiation of hADSCs.

Key Words: Differentiation, hADSCs, PKC, pPMA, Proliferation

INTRODUCTION

Multiple cell sources have been studied for repair and regeneration of defective tissues and organs. Mesenchymal stem cells (MSCs) can be obtained from the adult organism and have multi-lineage differentiation potential [1]. In the past decade, many clinical and pre-clinical studies have shown that bone-marrow-derived MSCs can be successfully used for the treatment of various diseases and disorders [2-4]. In addition to bone marrow, adipose tissue [5] is easily accessible, abundant, and a readily available source of MSCs.

Recent studies have shown that human adipose-tissue-derived stromal cells (hADSCs) can differentiate into mesenchymal and non-mesenchymal cell types, including adipocytes, osteoblasts, chondrocytes, muscles, neurons, hepatocytes, hematopoietic cells, and endothelial cells [6]. These favorable characteristics make human adipose tissue a promising alternative source of MSCs for regenerative medicine.

The majority of research using hADSCs has been performed using subcutaneous adipose tissue collected during cosmetic liposuction procedures. These analyses have tended to consider subcutaneous adipose tissue as a homogeneous entity despite evidence of heterogeneity in the biology of different subcutaneous adipose tissue depots in terms of fatty acid content [7], blood vessel density [8], and insulin sensitivity [9]. Moreover, the previous reports have shown differences between depots in adipogenic and osteogenic lineage potential [10] as well as apoptosis resistance and peroxisome proliferator activated receptor- γ expression [11].

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Corresponding to: Jin Sup Jung, Department of Physiology, School of Medicine, Pusan National University, Beomeo-ri, Mulgeum-eup, Yangsan 626-790, Korea. (Tel) 82-51-510-8071, (Fax) 82-51-510-8076, (E-mail) jsjung@pusan.ac.kr



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ABBREVIATIONS: hADSCs, human adipose-tissue-derived stromal cells; MSCs, mesenchymal stem cells; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; FACS, fluorescence-activated cell sorting.

Protein kinase C (PKC) has important roles in many physiological functions, including cell metabolism, proliferation, differentiation, cell migration, and apoptosis [12-15]. However, the role of PKC in osteogenic differentiation is still controversial. Activation of PKC- α has been shown to suppress osteogenic differentiation of mouse preosteoblastic MC3T3-E1 cells [16]. On the other hand, fibroblast growth factor receptor 2 (FGFR2) has been shown to increase osteoblast differentiation of C3H10T1/2 cells through the activation of extracellular regulated kinase (ERK) and PKC- α [17]. Liu et al. [18] reported that inhibition of the conventional PKCs/PKC- μ and activation of PKC- δ increased osteogenic differentiation of human bone-marrow-derived MSCs. In addition, the role of PKC in the differentiation of hADSCs has not yet been studied.

In this study, we isolated hADSCs from three different adipose tissue depots (upper abdomen, lower abdomen, and thigh) to evaluate their characteristics and determine whether they differ in their potential to differentiate into adipocytes or osteoblasts. We characterized hADSCs derived from these tissues by flow cytometry using standard cell surface markers and analyzed adipogenic and osteogenic differentiation. We also examined the effect of Phorbol 12-myristate 13-acetate (PMA), a PKC activator, on the differentiation of hADSCs into adipocytes or osteocytes.

METHODS

Isolation and culture of hADSCs from different adipose depots

Lipoaspirates were obtained from the upper abdomen, lower abdomen, and thigh from nine female donors undergoing elective liposuction surgery. Their mean age was 45 years ranged from 33 to 65 years. The isolation of hADSCs was performed as previously described with modification [19]. Briefly, under sterile conditions, lipoaspirated tissues were immediately placed in Dulbecco's modified Eagle's medium (DMEM) supplemented with 200 mg/mL streptomycin and 200 U/mL penicillin, were washed in PBS, and then digested with 0.075% collagenase type I in PBS for 1 h at 37°C in a shaking incubator. The pellet was collected by centrifugation at 400 g for 10 min and then treated with red blood cell lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA) for 10 min at room temperature. After centrifugation, the cellular pellet was filtered through a 40- μ m mesh filter to remove debris. The filtrate was centrifuged and the stromal vascular fraction (SVF) obtained was plated onto cell culture dishes in complete culture medium (DMEM containing 10% fetal bovine serum (FBS), 100 mg/mL streptomycin, 100 U/mL penicillin, 2 mM L-glutamine, 1 mg/mL amphotericin-B). Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. After 24 h, non-adherent cells were removed and adherent cells were washed twice with PBS. Confluent cells were trypsinized and expanded in culture dishes (passage 1, P1). Culture passages P4-P6 were used in all experiments. Each experiment was performed at least three times.

Flow cytometry

A total of 500,000 cells/sample were centrifuged at 1,000 g for 1 min at 4°C, re-suspended in 75 μ L of fluorescence-activated cell sorting (FACS) buffer (2% FBS in PBS) and

incubated on ice for 10 min. Cells were immunolabeled with 13 μ g/ μ L of primary antibody (CD31, CD34, CD45, CD73, CD90, or CD105; Millipore) for 30 min at 4°C with rotation, followed by centrifugation and three washes with FACS buffer. Secondary antibody (fluorescein isothiocyanate (FITC) anti-mouse; Molecular Probes) was added at a dilution of 1:400 and incubated at 4°C for 30 min. After washing, the resulting pellet was re-suspended in PBS and filtered through a 35- μ m nylon mesh to remove aggregated cells. Cell suspensions were kept on ice until cytometric analysis was performed using a FC500 flow cytometer (Beckman Coulter, Brea, CA, USA).

Proliferation assays

The proliferation of the hADSCs was assessed for 14 days using the CellTiter 96 Aqueous non-radioactive cell proliferation assay (Promega, Madison, WI, USA). hADSCs were plated using either control growth medium (CGM) alone, adipogenic induction medium (AIM) or osteogenic induction medium (OIM) without or with 10 nM PMA (Sigma, St. Louis, MO, USA). PMA was dissolved in dimethyl sulfoxide (DMSO) and were diluted at a final concentration of 10 nM with DMEM. DMSO with 0.01% (v/v) was used as a negative control.

At the time of seeding, the signal was assessed for all sources and normalized. At regular intervals (4, 7, 10, and 14 days), 20 μ L of CellTiter 96 Aqueous Assay Reagents (Promega) were added and incubated for 4 h at 37°C and 5% CO₂. The absorbance was recorded at 490 nm using a spectrophotometric plate reader and measurements were calculated by subtracting the average control absorbance (medium only) from the average cell absorbance.

Induction of osteogenic and adipogenic differentiation

hADSCs were grown to approximately 90% confluence and osteogenic differentiation was induced for 21 days in OIM (10% fetal bovine serum, 1% penicillin/streptomycin, 100 nM dexamethasone [Sigma-Aldrich, St. Louis, MO, USA], 50 μ M ascorbic-2-phosphate [Sigma-Aldrich] and 10 mM β -glycerophosphate [Sigma-Aldrich] in low glucose DMEM) with or without 10 nM PMA and evaluated for extracellular matrix calcification by staining with 2% Alizarin Red S (Sigma-Aldrich). Whole field images of 6-well culture plates were captured with a digital camera. For quantification, the stained dye was eluted with 10% acetic acid and 20% methanol in distilled water; the absorbance of supernatants was measured at 450 nm using a Multiskan FC reader (Thermo scientific, Shanghai, China) and compared between OIM with and OIM without 10 nM PMA.

For the induction of adipogenic differentiation, cells of 90% confluence were cultured in AIM (10% FBS, 1% penicillin/streptomycin, 1 μ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine [Sigma-Aldrich], insulin [Invitrogen, Carlsbad, CA, USA] 10 μ g/mL and 60 μ M indomethacin [Sigma-Aldrich] in low glucose DMEM) with or without 10 nM PMA for 14 days. Lipid droplets within differentiated adipocytes from hADSCs were observed using modified Oil Red O staining. Whole field light microscopy images were captured at 10 \times magnification. For quantification, the stained dye was eluted in 2-propanol; the absorbance of supernatants was measured at 500 nm using a Multiskan FC reader and compared between cells grown in AIM alone or AIM supplemented with 10 nM PMA.

Table 1. Primers used for real-time PCR amplification

Species	Gene	Sense	Anti-sense
Human	RUNX2	ACTGGCGCTGCAACAAGA	TCATCGTTACCCGCCATG
	ALP	CCATCCCACGTCTTCACATT	AGTGAAGGGCTTCTGTCTGTGT
	PPAR- γ	GAACAGATCCAGTGGCAGAT	TGAGGGAGTTGGAAGGCTCTT
	aP2	AGCACCATAACCTTAGATGGGG	CGTGGAAGTGACGCCTTTCA
	GAPDH	ATGGAAATCCCATCACCATCTT	CGCCCCACTTGATTTTGG

Real-time PCR

hADSCs were cultured for 7 days in CGM, OIM, and AIM with or without 10 nM PMA. Total RNA was collected using the initial steps of the TRIzol protocol (Invitrogen). After collecting the aqueous supernatant, the RNeasy column coupled with DNase set-based (Qiagen) protocol was followed. Reverse transcription was performed using 2 μ g total RNA, 25 μ g/mL oligo (dT)₁₂₋₁₈ primers, and 200 units of SuperScript II reverse transcriptase (Invitrogen). For real-time PCR, 5 ng cDNA was amplified using SYBR Green PCR Master Mix (Applied Biosystems) and 0.33 μ M each of forward and reverse primers on the ABI PRISM 7700 sequence detector system (Applied Biosystems). Primers for quantitative PCR were designed using the Primer Express software (Applied Biosystems) and synthesized at Cosmo Biotec (Korea). Analysis was performed using software (SDS version 2.1; Applied Biosystems) according to the manufacturer's instructions. Samples were normalized using the housekeeping gene *GAPDH*. Primers used for real-time PCR amplification are listed in Table 1. All samples were tested in triplicate, and mean values were used for quantification.

Statistical analysis

Data were presented as mean \pm standard deviation (SD). The Student's t-test was used to compare between two groups. One-way ANOVA analysis of variance was used to compare three groups. Differences with a p-value of <0.05 were considered statistically significant.

RESULTS

Immunophenotypic characterization of cultured hADSCs from different adipose tissue depots

Cultured hADSCs from upper abdomen, lower abdomen, and thigh regions were immunophenotypically characterized by FACS analysis. Subconfluent cultures of hADSCs (P4) were analyzed for CD73, CD90, CD105 (a surface marker of MSCs), CD31, CD34, and CD45 expression (a negative marker of endothelial and hematopoietic cells). The hADSCs obtained from three different regions were negative for the expression of CD31, CD34, and CD45 (Fig. 1A). The percentage of hADSCs expressing CD73 and CD90 was found to be similar among all hADSC sources. CD105 expression in hADSCs from the thigh region was significantly lower than that in hADSCs from upper and lower abdominal regions (Fig. 1B).

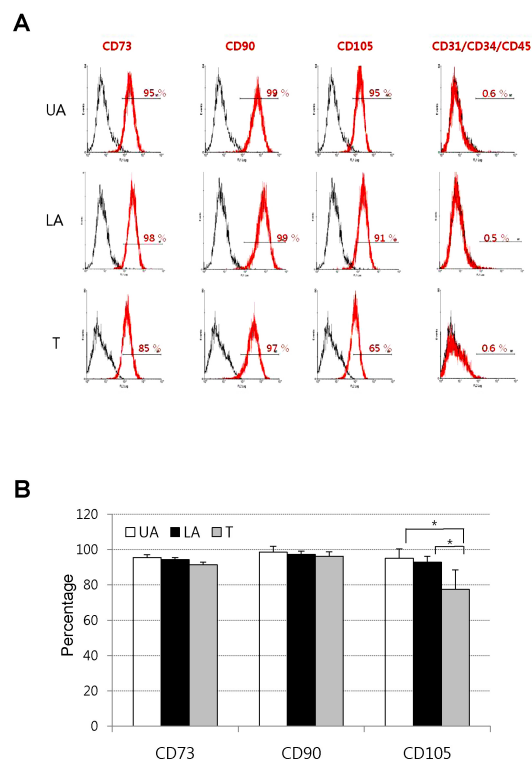


Fig. 1. Immunophenotypic characterization of ADSCs isolated from different subcutaneous tissues (UA, LA and T). (A) FACS analysis revealed positive for CD73, CD90 and CD105 and negative for CD31, CD34 and CD45. Back and red histograms represent control and specific antibody staining, respectively. (B) Histogram of CD73, CD90 and CD105 positive population in ADSCs isolated from different subcutaneous tissues (UA, LA and T). Data are expressed as mean \pm SD (n=3). *p<0.05.

Growth kinetics of hADSCs from different adipose tissue depots

To compare the growth of hADSCs obtained from different regions, the proliferation rate was assessed over 14 days using the CellTiter 96 Aqueous non-radioactive cell proliferation assay kit (Promega) in CGM, AIM, or OIM. The growth rate of hADSCs was increased in OIM, but decreased in AIM compared to the control group, CGM, although it has no significant difference according to tissue procurement sites (Fig. 2). The effect of PMA on the proliferation of hADSCs was determined in CGM, AIM, and OIM. Treatment of PMA also did not affect the proliferation rate, regardless of cell sources (Fig. 2).

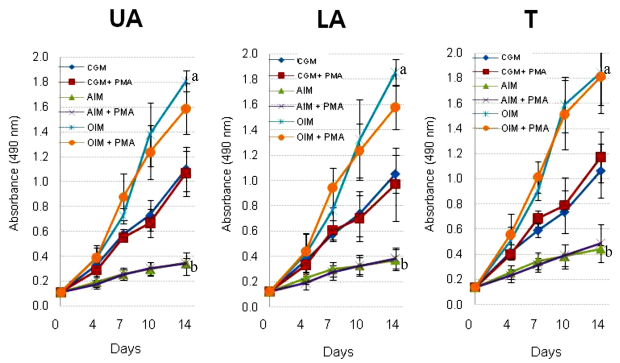


Fig. 2. Proliferation rate of hADSCs from different subcutaneous tissues. Cells were cultured in CGM)with or without 10 nM PMA, AIM with or without 10 nM PMA, and OIM with or without 10 nM PMA. Cell proliferation was evaluated on days 4, 7, 10, and 14 of culture. Data are indicated as the mean±SD (n=7). ^{a,b}p<0.05, significantly different from the CGM.

Osteogenic differentiation of hADSCs from different adipose tissue depots

To investigate the multipotentiality of hADSCs isolated from different subcutaneous regions, the osteogenic or adipogenic differentiation of P4-P6 hADSCs was induced. In OIM, cells proliferated rapidly, aggregated, and formed tightly packed colonies, with calcium deposition detected by Alizarin Red S staining over 3 weeks (Fig. 3A). Quantitative analysis of calcium deposition between hADSCs cultured in OIM versus CGM showed a five-fold increase in calcium deposition in hADSCs from thigh region compared with those from abdominal regions (Fig. 3B).

We determined the expression of osteogenesis-related genes in hADSCs from different adipose tissue depots. The expression level of *ALP* in undifferentiated hADSCs from the thigh region was significantly higher than that of undifferentiated hADSCs from abdominal regions. The ex-

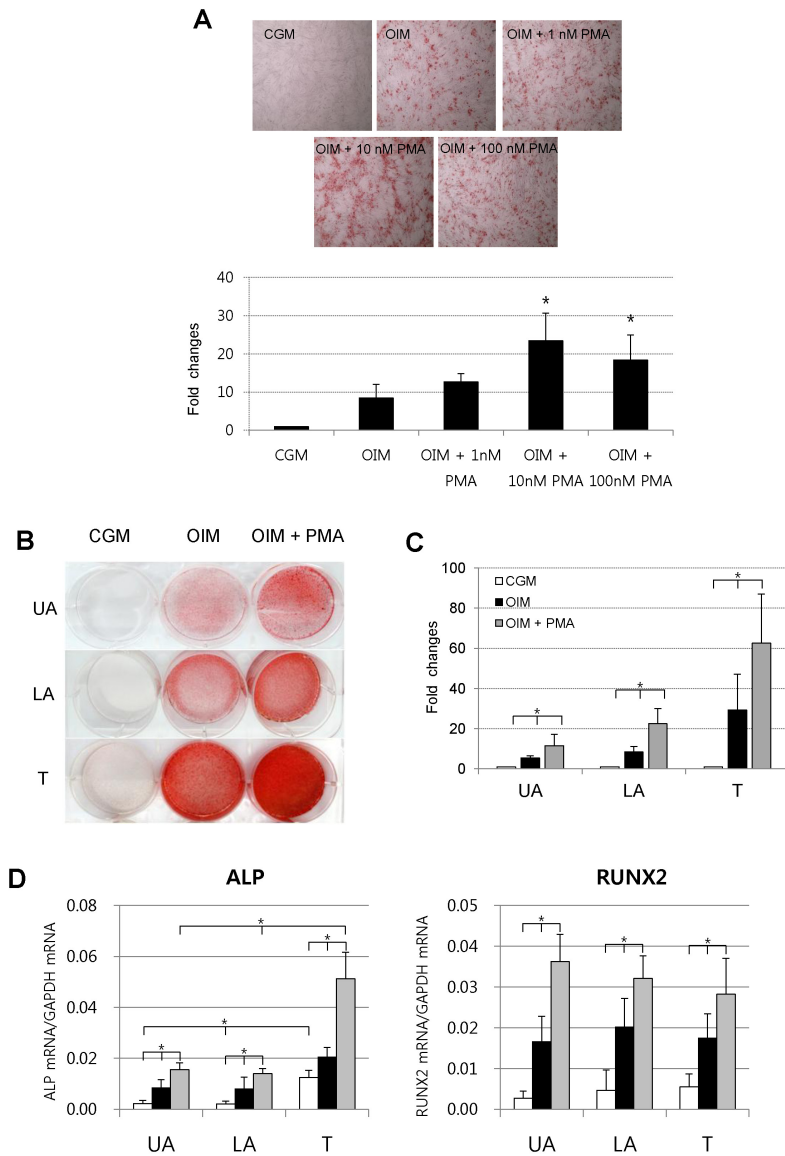


Fig. 3. Osteogenic differentiation of hADSCs from different subcutaneous tissues. (A) Effect of PMA on osteogenic differentiation of hADSCs from LA. hADSCs were cultured in CGM or OIM with or without PMA (1, 10 and 100 nM) for 14 days and then stained with Alizarin red S. Quantitative data were obtained from destained Alizarin red S absorbance. Data are indicated as the mean±SD (n=3). *p <0.05, significantly different from OIM. (B) hADSCs from the UA, LA, and T regions were cultured in CGM or OIM with or without 10 nM PMA for 14 days and then stained with Alizarin red S. (C) Quantitative data obtained from destained Alizarin red S absorbance. (D) Quantitative analysis of the expression of osteogenesis-related genes. hADSCs from UA, LA, and T regions were treated with CGM or OIM with or without 10 nM PMA for 7 days. Total RNA was extracted and the expression levels of *ALP* and *RUNX2* were analyzed by real-time PCR. White, black, and grey bars represent CGM, OIM, and OIM with 10 nM PMA, respectively. Data are expressed as mean±SD (n=9). *p<0.05.

pression level of *RUNX2* in undifferentiated hADSCs was no different among sources. The expression levels of *ALP* and *RUNX2* increased according to osteogenic differentiation in hADSCs from different depots (Fig. 3C).

Adipogenic differentiation of hADSCs from different adipose tissue depots

When cultured in AIM, a predominance of lipid-droplet formation was observed for all hADSCs by Oil Red O staining (Fig. 4A). Quantitative analysis of lipid-droplet formation between hADSCs cultured in AIM versus CGM revealed that there was no significant difference among hADSCs from different regions (Fig. 4B).

The expression levels of *AP2* and *PPAR- γ* genes (marker genes for adipogenic differentiation) were examined using real-time PCR. Expressions of both genes were significantly increased in AIM compared to the CGM. However, expression of these genes had no significant difference among different depots in both CGM and AIM (Fig. 4C).

Effects of PMA on differentiation of hADSCs

To investigate whether adipogenic or osteogenic differentiation of hADSCs is affected by PKC signaling, we treated hADSCs with PMA for 14 days in AIM or OIM. To determine the optimal concentration of PMA, we determined the effect of various concentrations of PMA on osteogenic differentiation of hADSCs that were isolated from lower abdomen. The treatment of PMA increased osteogenic differentiation of hADSCs and 10 nM PMA showed the maximal effect (Fig. 3A). Therefore, we used 10 nM PMA in the following experiments. Activation of PKC resulted in stimulation of osteogenic differentiation of hADSCs (Fig. 3A and B) and inhibition of adipogenic differentiation of hADSCs (Fig. 4A and B).

The effect of PMA on expression of adipogenesis-related and osteogenesis-related genes was examined using real-time PCR. Both *ALP* and *RUNX2* genes were highly expressed in hADSCs cultured in OIM compared to CGM. In addition, the expression levels of these genes were increased by PMA treatment in all hADSCs from different

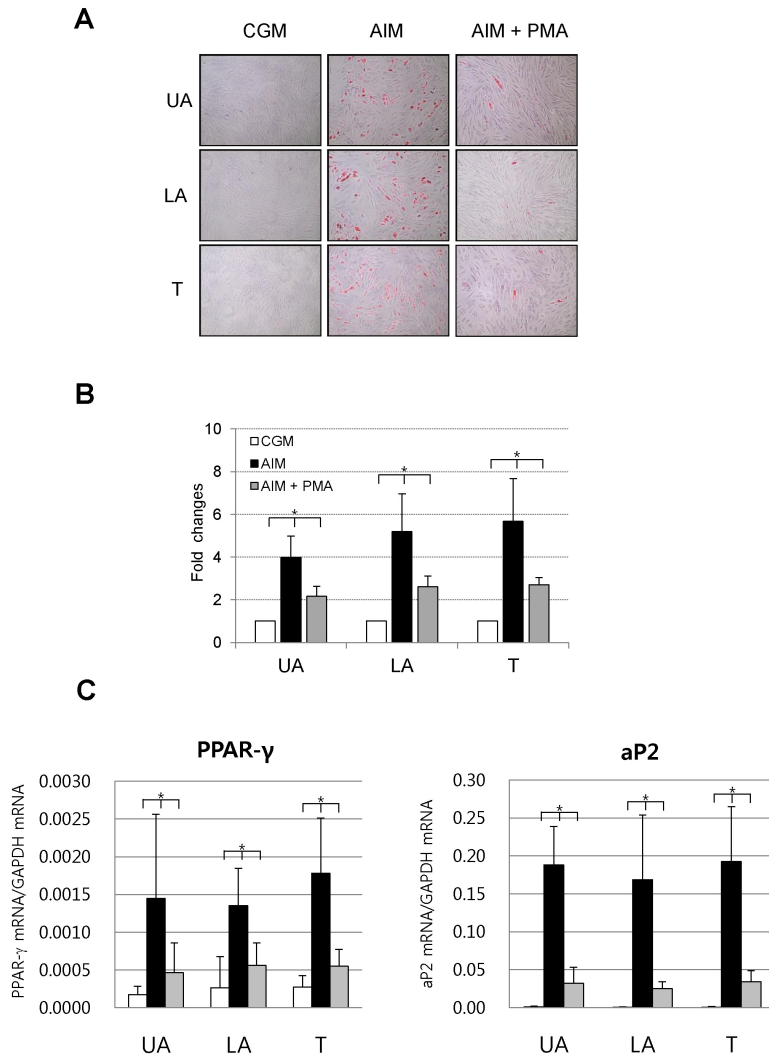


Fig. 4. Adipogenic differentiation of hADSCs from different subcutaneous tissues. (A) hADSCs from UA, LA, and T regions were cultured in control growth medium (CGM) or adipogenic induction medium (AIM) with or without 10 nM phorbol 12-myristate 13-acetate (PMA) for 14 days then stained with Oil Red O. (B) Quantitative data obtained from lipid droplets. (C) Quantitative analysis of the expression of adipogenesis-related genes. hADSCs from UA, LA and T regions were treated with CGM or AIM with or without 10 nM PMA for 7 days. Total RNA was extracted and the expression of *PPARG* and *AP2* was analyzed by real-time PCR. White, black, and grey bars represent CGM, AIM, and AIM with 10 nM PMA, respectively. Data are expressed as mean \pm SD (n=9). *p<0.05.

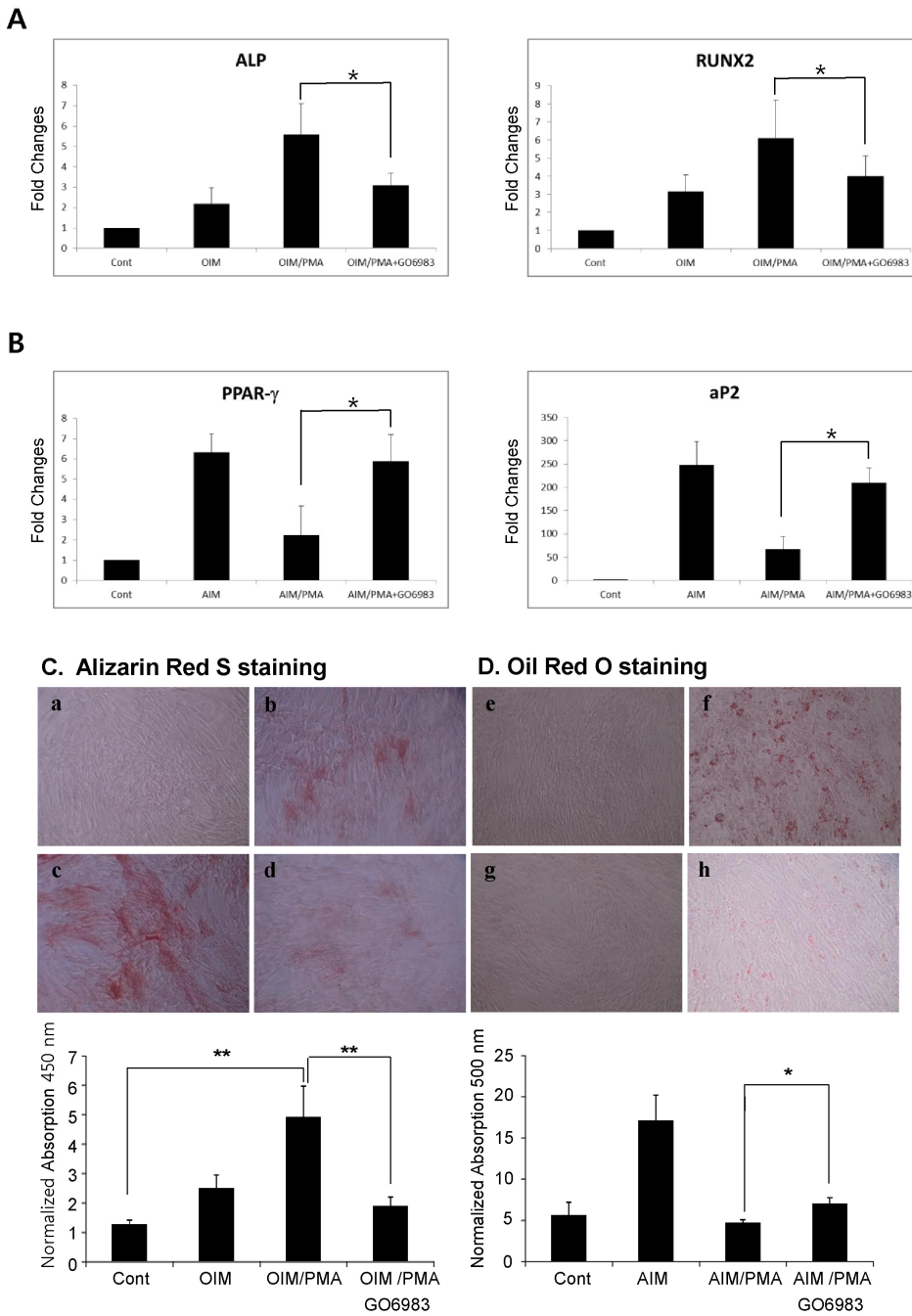


Fig. 5. The effect of PKC inhibitor, Go6983, on osteogenesis and adipogenesis of hADSCs by PMA. After pretreatment of hADSCs from thigh regions with Go6983 for 1 hour, cells were cultured in the OIM or AIM with or without 10 nM PMA. After 7 days of culture, total RNA was extracted and the expression levels of ALP, RUNX2 (A), PPAR and aP2 (B) were analyzed by real-time PCR. After 3 weeks of culture, the cells were stained with Alizarin red S staining for osteogenic differentiation (C) and Oil-red O staining for adipogenic differentiation (D). The degree of Alizarin red S for quantification of mineral deposition and Oil-Red O for quantification of lipid were measured at 450 nm and 500 nm with a 96-well plate reader, respectively. Data are expressed as mean±SD (n=6). a) CGM, b) OIM, c) OIM+PMA, d) OIM+PMA+Go6983, e) CGM, f) AIM, g) AIM+PMA, and h) AIM+PMA+Go6983. *p<0.05 and **p<0.01.

depots. However, the expression levels of these genes after PMA treatment had no significant difference among different depots (Fig. 3C). On the other hand, the expressions of *AP2* and *PPARG* in AIM were significantly increased compared those in CGM, but their expressions were down-regulated by PMA treatment in all hADSCs from different depots (Fig. 4C). There was no significant difference in the magnitude of expression change induced by PMA treatment among hADSCs from upper abdomen, lower abdomen, and thigh regions.

We then examined the effect of PKC inhibitor on PMA-induced changes in osteogenesis and adipogenesis of hADSCs.

Go6983, a general PKC inhibitor [20], was added before one hour adding PMA in OIM or AIM culture of hADSCs from thigh regions. Pretreatment with Go6983 inhibited PMA-induced increase in *ALP* and *RUNX2* expression and the alizarin red S staining (Fig. 5A, 5C). Go6983 pretreatment significantly increased the reduced *AP2* and *PPARG* expression and the oil-red O staining induced by PMA (Fig. 5B, 5D).

DISCUSSION

In this study, we have investigated whether proliferation and differentiation of cultured hADSCs are affected by the region of adipose tissue procurement (upper abdomen, lower abdomen, or thigh). The growth rate of cultured hADSCs in CGM was similar, irrespective of the tissue procurement site. Although OIM and AIM affected hADSC proliferation, their effects were similar among hADSCs from all three sources. These findings indicate that hADSC growth rate does not vary according to the source of subcutaneous tissue.

Flow cytometric analysis showed that the proportion of hADSCs expressing CD73 and CD90 was similar among hADSCs from all regions, whereas the proportion of CD105-positive hADSCs from the thigh region was lower than that of hADSCs from the upper abdomen and lower abdomen. hADSCs from all regions had similar adipogenic differentiation potential, but osteogenic differentiation potential was significantly different between hADSCs from the abdominal and thigh regions. This finding is consistent with the results of a previous study [20]. Levi et al. [10] showed that FACS-sorted CD105^{low} hADSCs had better *in vitro* osteogenic differentiation potential and *in vivo* bone regeneration than either CD105^{high} or unsorted cells through reduction of TGF- β 1 signaling.

ALP and *RUNX2* are expressed during osteogenic differentiation and are the most widely recognized markers of osteogenic differentiation [20-22]. In this study, the expression levels of ALP and *RUNX2* were increased during osteogenic differentiation in all hADSCs. The expression level of ALP in un-differentiated hADSCs from the thigh region was significantly higher than that in hADSCs from abdominal regions. Thus, the superiority of osteogenic differentiation in hADSCs from the thigh region may be related to the lower level of CD105 expression and higher level of ALP expression.

In this study, we found that 10 nM PMA treatment increased the osteogenic differentiation of hADSCs and significantly suppressed the formation of oil droplets and expression levels of *PPARG* and *AP2* mRNA; the effects of PMA did not show any regional variation. These effects were completely blocked by the treatment with the PKC inhibitor Go6983, which inhibits all PKC isoforms [23], indicating that the effect of PMA on adipogenesis and osteogenesis is mediated by the activation of PKC pathways.

The effect of PKC on osteogenic and adipogenic differentiation of MSCs is still controversial. Nakura et al. showed that PKC- α suppressed osteogenic differentiation of mouse preosteoblastic MC3T3-E1 cells [16]. By contrast, Liang et al. reported that PMA increased osteogenic differentiation of mouse osteoblasts MC3T3-E1 [24], and Richardson et al. [25] showed that oxysterol-induced osteoblastic differentiation in M2-10B4 MSCs and C3H10T1/2 embryonic fibroblastic cells was inhibited by PKC inhibitors. The PKC family comprises at least 11 serine/threonine kinases. PKC isoforms can be classified into three classes: classical, novel, and atypical [26,27]. Classical (α , β and γ) and novel PKCs (δ , ϵ , η , θ and μ) rely on phospholipids for activation, whereas atypical PKCs (ζ , ι , and λ) do not require these lipids. Several studies have indicated that cellular response to PKC activation varies according to PKC isoforms. Artemenko et al. [28] reported that the anti-adipogenic effect of PDGF is mediated by the activation of PKC- α in murine 3T3-L1 preadipocyte and human preadipocytes.

Webb et al. [27] showed that Protein kinase C- ϵ promotes adipogenic commitment and is essential for terminal differentiation of 3T3-F442A preadipocytes. Zhou et al. [29] reported that PKC- β is important for the induction of adipogenesis, while PKC- δ has an inhibitory role in adipogenesis in 3T3-L1 preadipocytes. In human MSCs, activation of the conventional PKCs/PKC- μ inhibited osteogenic differentiation, and activation of PKC- δ promoted osteogenic differentiation of human bone marrow derived MSCs *in vitro* [30]. Thus, the differences in PKC-induced action on the osteogenic and adipogenic differentiation of hADSCs observed in other studies might be related to the different levels of PKC isoforms.

In conclusion, our finding that the osteogenic potential of hADSCs from the thigh region is significantly greater than that of hADSCs from the upper abdomen and lower abdomen indicates that the thigh region might be a suitable source of hADSCs for bone regenerative medicine applications. In addition, PMA increases osteogenic differentiation potential and suppresses adipogenic differentiation, suggesting that the PKC signaling pathway might be involved in the adipogenic and osteogenic differentiation of hADSCs. Further studies on the regulatory mechanism of PKC could provide important insight regarding the control of differentiation of hADSCs.

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